Styrene oxidation to styrene oxide in human erythrocytes is catalyzed by oxyhemoglobin

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Summary. Oxygenated human erythrocytes catalyzed the oxidation of styrene to styrene oxide. This reaction was inhibited by CO but not by superoxide dismutase, catalase and scavengers of hydroxyl radicals. In partially deoxygenated erythrocytes styrene oxidation showed a linear relationship with the molar fraction of oxyhemoglobin. These data indicate that oxyhemoglobin and not free oxygen radicals are involved in styrene oxidation.

Hemoglobin has been implicated as a catalyst of various reactions such as lipid peroxidation[2], the decarboxylation of dopa by lysed erythrocytes[3,4], the dealkylation of some aromatic N,N-dimethylamine-N-oxides[5,6] and the hydroxylation of aniline[7-9]. Hemoglobin, however, is not considered to be an enzyme, probably because it is known that iron chelates can replace hemoglobin and that the required concentrations of this hemoprotein are generally high[9].

It has been reported that in the oxyhemoglobin molecule the oxygen is in a partially activated form[10,11] and reactive oxygen intermediates can be released during hemoglobin autoxidation[12]. In a previous report we showed that human erythrocytes were able to catalyze styrene oxidation to styrene oxide[13]. Here we report a further characterization of this reaction showing that it does not depend on free reactive oxygen intermediates but is dependent on the amount of oxyhemoglobin in the red blood cells.

Materials and methods. Human venous blood suitable for transfusion (with 0.68% citrate w/v as anticoagulant) was obtained from AVIS (Associazione Volontari Italiani del Sangue); PBS (phosphate buffered saline without Ca^{++} and Mg^{++}) was purchased from Eurobio, Paris. Superoxide dismutase, type I and catalase were obtained from Sigma (St. Louis, Miss., USA). Erythrocytes were isolated according to a previously published procedure[14]. To obtain the cell lysate, washed erythrocytes were rapidly frozen in acetone/CO_2 and thawed 4 times at room temperature; the sample was then centrifuged at 10,000 × g for 10 min. Erythrocytes were deoxygenated at 37 °C in small, tightly sealed pyrex flasks[15], previously washed with N_2 when full deoxygenation was required. Partial deoxygenation was carried out in a tonometer (Instrumentation Laboratories, Paderno Dugnano, Italy) under a continuous flow of an appropriate N_2/O_2 ratio. The concentration of oxyhemoglobin was determined at the end of tonometry (25 min) by a micromethod[16].

Erythrocytes (0.114 ml, $500 \cdot 10^6$ cells/ml) were incubated with styrene dissolved in acetonitrile (0.90 M) in PBS (pH 7.4) in a final volume of 1 ml (hemoglobin concentration 0.25 mM). After 30 min of incubation at 37 °C, under N_2 in

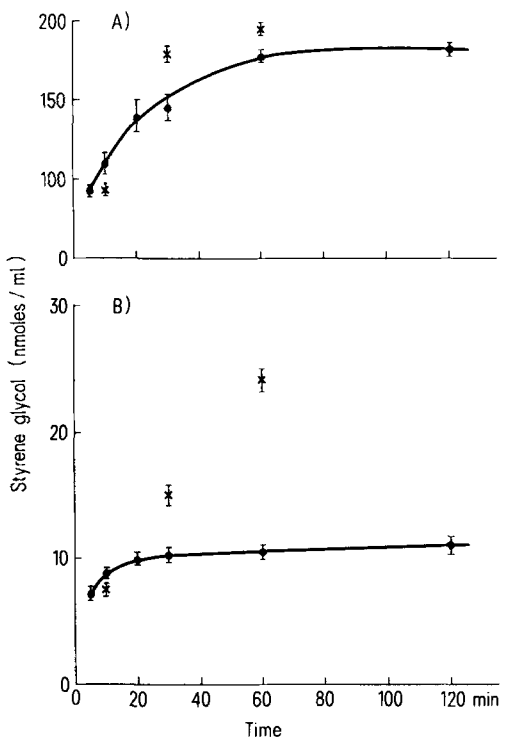

Figure 1. Time course of styrene oxidation to styrene oxide in human erythrocytes (×) and cell lysate (○). Red blood cells and lysate preparation was as described in 'Materials and methods'. Intact cells and lysate were incubated with styrene 50 mM (panel A) and 0.8 mM (panel B). Brackets represent the mean ± SEM.

Styrene oxidation to styrene oxide in washed human erythrocytes

System	Styrene glycol[a] nmoles/30 min/ml
Erythrocytes	130.0± 6.0
+ CO[b]	29.2± 1.0
+ Superoxide dismutase (50 units/ml)	117.2± 1.4
+ Catalase (1750 units/ml)	158.3± 16.2
+ Tryptophan (2 mM)	104.0± 5.2
+ Mannitol (20 mM)	126.0± 6.3
+ Dimethyl sulfoxide (280 mM)	150.0± 8.0

Styrene concentration in the incubation mixture was 50 mM. The data represent the mean ± SEM. [a]The styrene oxide formed enzymatically was chemically converted to styrene glycol (see Materials and methods). [b]CO was bubbled into the incubation mixture for 1 min at a flow rate of 50 ml/min before addition of styrene.

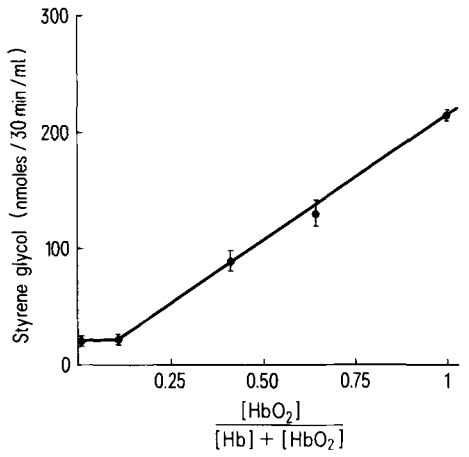

Figure 2. Relationship between the molar fraction of oxyhemoglobin and styrene oxidation to styrene oxide in human blood erythrocytes.

sealed flasks for deoxygenated samples, the reaction was stopped with 0.4 M of 0.6 N H_2SO_4.

Styrene oxide formation was evaluated according to a previously described method[17-19]. In this procedure at the end of incubation the styrene oxide formed is quantitatively chemically hydrated by overnight incubation with H_2SO_4 to the glycol, which is more suitable for gas chromatographic analysis. Styrene glycol is quantitatively determined by a sensitive gas chromatographic procedure using an electron capture detector[20].

Results and discussion. We have already shown that human erythrocytes are able to catalyze styrene oxidation to styrene oxide[13] and that this reaction was supported by methemoglobin and H_2O_2 (Cantoni et al.[21]). It is known that the oxygen in oxyhemoglobin is in a partially activated form[10,11] and that iron chelates in the presence of H_2O_2 can generate reactive oxygen intermediates[22,23]; the superoxide anion (O_2^-) can also be released in erythrocytes during autoxidation of hemoglobin[12]. The table shows that CO almost completely inhibited styrene oxidation, probably by O_2 displacement, suggesting an important role for hemoglobin in this reaction. Superoxide dismutase and catalase had no effect, indicating that O_2^- and H_2O_2 were not directly involved. With the styrene concentration used for this experiment (50 mM) a 100% cell lysis occurred in 5 min (data not shown) therefore these enzymes would be able to penetrate to the site where styrene oxidation occurs. Likewise, scavengers of hydroxyl radicals such as tryptophan[22,24], mannitol and dimethylsulfoxide[24,25] did not inhibit styrene oxidation to styrene oxide.

Figure 1 reports the time course of styrene oxidation with 2 different styrene concentrations in intact cells and in cell lysate. With the higher styrene concentration (50 mM), able to cause cell lysis, the time courses in both systems almost overlap (panel A). At the lower styrene concentration (0.8 mM) (panel B), which does not cause cell lysis, intact cells are more active than lysate probably because the oxyhemoglobin concentration is higher inside the cells than in the lysate, although the total amount of oxyhemoglobin was the same in both samples. It has been shown that O_2 release increases with the concentration of oxyhemoglobin[27].

Incubation of partially deoxygenated erythrocytes with styrene showed a linear relationship between styrene oxidation and the molar fraction of oxyhemoglobin contained in the red blood cells (fig. 2).

These findings seem to indicate that free reactive oxygen intermediates, able to oxidize organic molecules[28-30], are not directly involved in styrene oxidation in erythrocytes, but that this reaction is effected by oxyhemoglobin.

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Interference of azide in the estimation of total lipids by means of the sulphophosphovanillin method

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Summary. The commonly used bacteriostaticum sodium azide interacts with double bonds of lipids, causing an underestimation of lipid concentration when lipids are assayed in its presence. This can be circumvented by extraction of lipids prior to the assay.

Part of our research on reproduction physiology of the starfish *Asterias rubens* (L.) is concerned with the nature of materials transported from the storage organs (pyloric caeca) to the gonads. It was supposed earlier[2] that these materials are transported in a complex form as a glycolipoprotein.

The amounts of lipid and carbohydrate in fractions obtained by gel filtration of pyloric caeca and gonad homoge-